

Original Article

CREB is upregulated in hepatic encephalopathy and contributes to the neuroprotection against neuroinflammation

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Abstract: Background: The cyclic adenosine monophosphate (cAMP) response element (CRE)-binding protein (CREB) is the key transcription factor in the brain which strengthens cell connection. This study was intended to determine whether CREB affect neurological decline in a hepatic encephalopathy animal model under trial conditions. Methods: Hepatic encephalopathy was induced in C57BL/6 mice via intraperitoneal injection of azoxymethane (AOM) in the presence or absence of 2-naphthol AS-E phosphate (KG-501). Mice were monitored for neurological decline and time to coma was recorded. The mice cortex were collected until coma and were used for real-time PCR and immunoblot analysis to assess the consequences on proinflammatory cytokine expression and the phosphorylation levels of the main factors in signaling pathway. Results: The expression of CREB was significantly elevated in AOM-treated mice cortex. But in KG-501 prior injection to AOM-treated mice cortex, the concentrations of chemokine (C-C motif) ligand 2 (CCL2) and CREB were significantly decreased. KG-501 significantly promoted the neurological outcomes of AOM-treated mice, reduced phosphorylation of ERK and the concentration of cAMP in cortex. Conclusions: These findings suggested that CREB was elevated following acute liver injury and CREB was involved in neurological decline associated with hepatic encephalopathy and the cAMP/ERK signaling pathway.

Keywords: CREB, azoxymethane, neuroinflammation, chemokine ligand 2, ERK signaling pathway, cAMP

Introduction

Hepatic encephalopathy is a serious neuropathological state that arises as a part of a multi-organ manifestation of liver failure with the potential to affect health-related quality of life, clinical management strategies, priority for liver transplantation, and patient survival. It is defined as a metabolically induced, potentially reversible, functional disturbance of the brain [1]. The neuropathological features of hepatic encephalopathy primarily include changes in the morphology and function of cells of the glial (neuronal) lineage and have led to the suggestion that hepatic encephalopathy is a primary gliopathy [2]. Associated with these differences in neurological presentation, the disease processes that generate hepatic encephalopathy due to acute and chronic liver failure differed significantly [3]. Increased inflammation has been demonstrated to lead to the increased progression of hepatic encephalopathy and

worse outcomes for patients with liver failure [4]. It was also found that proinflammatory cytokines were elevated and derived from increased production in the brain [5]. Although prediction of survival in acute liver failure has been extensively investigated in many studies, predictive factors for progression to deep hepatic encephalopathy have not been fully elucidated.

The cyclic adenosine monophosphate (cAMP) response element (CRE)-binding protein (CREB) is one of the first transcription factors purified by DNA affinity column using the CRE of the somatostatin gene [6]. It was well established that transcription factor CREB regulates learning and memory [7]. Extracellular stimuli elicit changes in gene expression in target cells by activating intracellular protein kinase cascades that CREB phosphorylate [8]. Additionally, as a nuclear regulatory factor, CREB implements the function of regulating the transcription by auto-phosphorylation. It is an action point of multiple

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intracellular signal transduction pathway, which plays important roles in adjusting of normal learning, memory and emotions maintain [9]. Previous study has reported that activation of CREB dependent transcription in reactive astrocytes prevented secondary injury in cerebral cortex after experimental traumatic brain injury [10]. Besides, the deregulation of CREB dependent transcription may contribute to brain pathologies including addiction epilepsy and neurodegeneration [11-13]. While the etiology of hepatic encephalopathy has not been clearly elucidated yet, it is believed that multiple factors are involved [14]. To date, no data existed concerning the role of CREB in the progression of hepatic encephalopathy induced by acute liver failure.

Therefore the aim of this study was to assess the expression of CREB and its receptors in a rodent model of hepatic encephalopathy, and also to determine how CREB contributes to the neurological decline observed in this disorder, which may help to identify one of the signals involved in initiating the proinflammatory response occurred during hepatic encephalopathy.

Material and method

Murine azoxymethane (AOM) model of hepatic encephalopathy

Adult male C57BL/6 mice (20 to 25 g; Charles River Laboratories, Wilmington, MA, USA) were used in all *in vivo* experiments. Mice were given free access to get water and rodent chow and were housed in constant temperature, humidity, and 12 h light-dark cycling. AOM (Sigma) was used to do single intraperitoneal injection to induce acute liver failure and hepatic encephalopathy of mice. In parallel, CREB activity was reduced via intracerebroventricular infusion of 2-Naphthol AS-E Phosphate (KG-501, Sigma) 3 days prior to injection of AOM [15]. The mice were randomly divided into 3 groups, namely vehicle, AOM and AOM + KG-501 groups. The mice in AOM group were intraperitoneal injected with 100 mg/kg AOM. For mice in vehicle group, the same volume of physiological saline was used to do single intraperitoneal injection. In the AOM + KG-501 group, the mice were treated with AOM (100 mg/kg, intraperitoneal) and intracerebroventricular infusion of KG-501 (10, 20, or 50 μ M). The mice in vehicle and AOM

group were infused with the same volume of dimethyl sulfoxide (DMSO, 1% for final concentration, Sigma) at the same time point.

After injection, mice were placed on heating pads set to 37°C to ensure they remained normothermic. To ensure the euglycemia and hydration, hydrogel and rodent chow were placed on cage floors to ensure that they could access to food and hydration. After 12 h and every 4 h following, mice were injected subcutaneously with 5% dextrose in 250 μ l of saline. Mice were removed from the study if they underwent a 20% weight loss [16]. All animal experiments performed were approved by the local IACUC regulations on animal experiments and comply with ARRIVE guidelines.

Neurological assessment

Neurological functioning was assessed by measuring the pinna reflex, corneal reflex, tail flexion reflex, escape response reflex, righting reflex, and ataxia, which were assessed and scored on a scale of 0 (no reflex) to 2 (intact reflex). The neurological score at each time point was defined as the summation of these reflex scores. In addition, time to coma (defined as a loss of all reflexes) was recorded [17]. Prior to coma, three neurological stages were defined based upon reflex measures: A pre-neurological decline stage (Pre) in which mice showed reduced activity but no deficits in reflexes or ataxia. The mice reached minor neurological decline (Minor) once they developed delaying in any measured reflex and the presence of mild ataxia, such as having difficulty walking across a metal cage lid. Major neurological decline (Major) occurs when there were severe deficits in all reflexes with the presence of significant ataxia [16]. Fresh mice brain cortexes were stripped carefully and flash frozen at the identified stages of neurological decline and coma (loss of corneal and righting reflexes) for further analysis.

Real-time PCR

Total RNA was extracted with Trizol (Invitrogen, USA) from flash frozen tissue and reversely transcribed by MultiScribe RT kit (Applied Biosystems, Foster City, California, USA) according to the manufacturer's instructions. All reverse transcriptase reactions were run in a master cycler gradient (Eppendorf, Germany)

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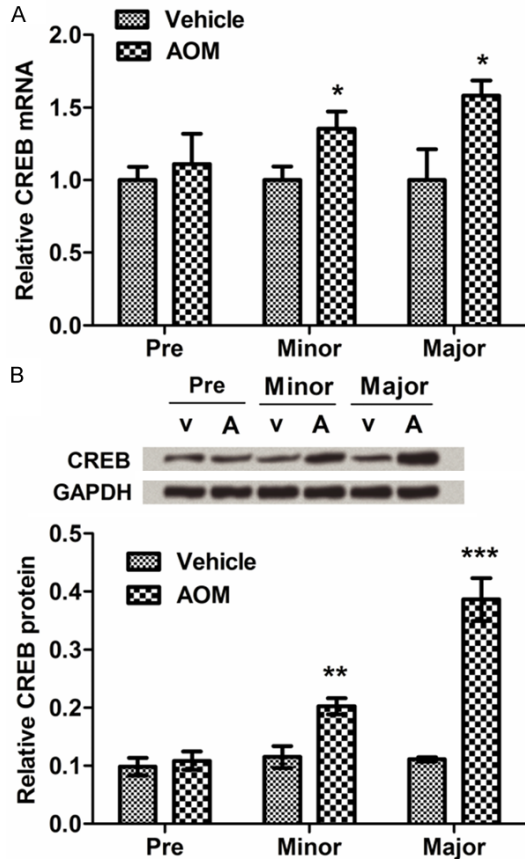


Figure 1. CREB was upregulated in the cortex of AOM induced mice. A. Cortical CREB mRNA expression during the time course of AOM-induced neurological decline (n = 3). B. CREB protein immunoblots and quantification in the cortex during the time course of AOM induced mice neurological decline (n = 3). GAPDH acted as an internal control. CREB, cyclic adenosine monophosphate response element binding protein; AOM, azoxymethane; V, vehicle group; A, AOM group; *P < 0.05, **P < 0.01 and ***P < 0.001 when compared to vehicle-treated mice.

using the SYBR® Green PCR Master mix (Super Array, USA) according to the manufacturer's instructions. The real-time PCR was performed in triplicate, including no template controls [18]. All primers were synthesized by Gene-Pharma (Shanghai, China). A $\Delta\Delta CT$ analysis was performed using vehicle-treated tissue as control for subsequent experiments [19]. Data were expressed as relative mRNA levels \pm Standard Error of Mean (SEM).

Immunoblotting

Western blots were loaded with 10-20 μ g of protein diluted in Laemmli buffer (Santa Cruz, USA) per each tissue sample (coma mouse cor-

tex induced with AOM, treated with 50 μ M KG-501, and loaded in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Western blotting was performed on nitrocellulose membranes using standard techniques, as described previously [20]. Specific antibodies against CREB (sc3-77154), extracellular signal-regulated kinase (ERK, sc514302), phosphorylated ERK (p-ERK, sc7383), c-Jun N-terminal kinase (JNK, sc7-345), phosphorylated JNK (p-JNK, sc6254), GAPDH (sc365062) (Santa Cruz biotech) and chemokine (C-C motif) ligand 2 (CCL2, ab18-6421, Abcam, Cambridge, MA, USA) were used along with appropriate fluorescent secondary antibodies (Santa Cruz biotech). All treatment groups were expressed as changes of fluorescent band intensity of target antibody to GAPDH, which was used as a loading control relative to vehicle or control groups. The values of vehicle or control groups were used as a baseline and set to a relative protein expression value of one. The band intensity quantification was performed using Image J software (National Institutes of Health, Bethesda, MD). Data for all experiments were expressed as mean relative protein \pm SEM.

Quantitative cAMP measurement

The cAMP analyses samples were prepped from coma mouse cortex induced by AOM and treated with 50 μ M KG-501 via homogenization in lysis buffer supplemented with protease inhibitor cocktail. Protein concentrations were performed using the cAMP Activity Assay Kit from Bio Vision (Enzo life Sciences, NY, USA) according to the manufacturer's instructions. The fluorescence signal was detected by measuring absorbance at 450 nm.

Statistical analyses

All statistical analyses were performed using Graphpad Prism 5 (Graphpad Software, La Jolla, CA, USA). Results were expressed as mean \pm SEM. For data that passed normality tests, significance was established using the Student's t-test between two groups. The differences between three or more groups were compared followed by the appropriate *post hoc* test. If data were non-parametric, a Kruskal-Wallis test was conducted. Differences were considered significantly when the P value was less than 0.05.

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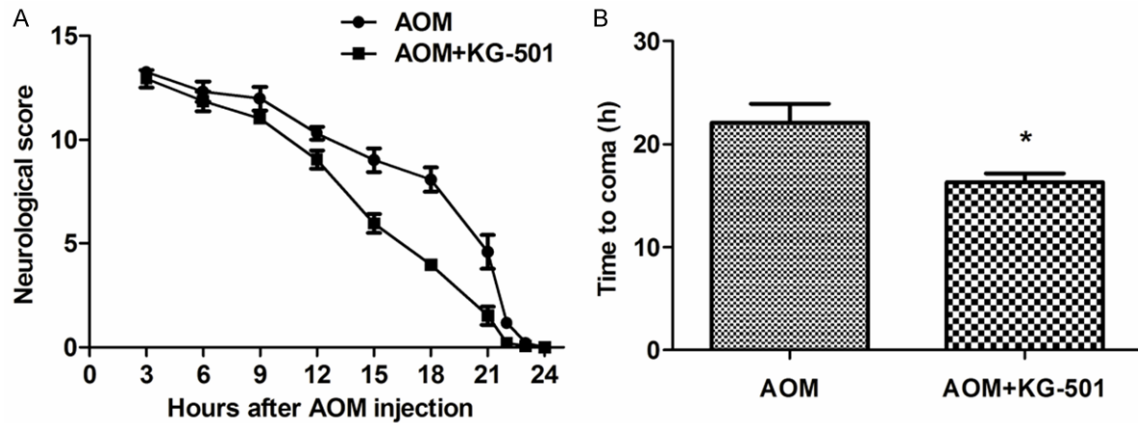


Figure 2. Inhibition of CREB was facilitation for AOM induced neurological decline in the progression of hepatic encephalopathy. A. Neurological score of mice in AOM and AOM + KG-501 group ($n = 4$). Neurological score calculations are outlined in the methods section. B. Time to coma of mice in AOM and AOM + KG-501 group ($n = 4$). CREB, cyclic adenosine monophosphate response element binding protein; AOM, azoxymethane; KG-501, CREB inhibitor; * $P < 0.05$ when compared to AOM-treated mice.

Results

CREB was elevated following AOM-induced hepatic encephalopathy

In order to examine the regulation of CREB during hepatic encephalopathy, mice were injected with AOM and the CREB expression level was assessed at the Pre, Minor, and Major stage. CREB mRNA expression in the cortex was found to be significantly elevated ($P < 0.05$) following the development of neurological dysfunction and increased throughout the time course of this experiment (Figure 1A). As shown in Figure 1B, cortical CREB protein expression demonstrated more obvious trend at minor and major stages of neurological decline ($P < 0.01$ and $P < 0.001$). These data demonstrated that CREB was up-regulated in the cortex of AOM-treated mice.

Inhibition of CREB was facilitation for AOM induced neurological decline

As CREB concentrations were significantly upregulated, the function of CREB activity in AOM-induced acute hepatic failure model was investigated. KG-501 (50 μM), antagonist against the CREB, was intracerebroventricularly infused prior to AOM administration. Pretreatment with KG-501 was found to significantly hasten the neurological decline and shorten the time taken to reach coma than AOM-treated mice ($P < 0.05$; Figure 2A and 2B). The nervous damage indicated that the decreased time

taken to reach coma in mice infused with KG-501 was probably due to subdued of neuroprotective mechanisms.

CREB inhibition elevated neuroinflammation caused by AOM-induced hepatic encephalopathy

Next, we assessed the role of CREB from cerebral cortex in mitigating the neuroinflammatory response which might contribute to inhibit the development of hepatic encephalopathy. The expressions of CREB and CCL2 in AOM-treated mice with increasing concentrations of KG-501 intracerebroventricular infusion (10, 20 and 50 μM) were analyzed. The CREB expressions in mice cortex of AOM + KG-501 group were significantly suppressed by 20 μM and 50 μM of KG-501, when compared to AOM group ($P < 0.05$ or $P < 0.01$; Figure 3A). And in Figure 3B, the expression level of CCL2, a chemokine previously shown to promote hepatic encephalopathy pathology, was significantly suppressed ($P < 0.01$) in 50 μM of KG-501 injection. These down-regulations of CREB and CCL2 further confirmed CREB was protective from AOM-induced neurological decline.

CREB inhibition reduced ERK activation and cAMP concentration in AOM-induced mice

As treatment with KG-501 was able to elevate neuroinflammation, validations that the treatment of reducing CREB-dependent signaling were required. The phosphorylation levels of

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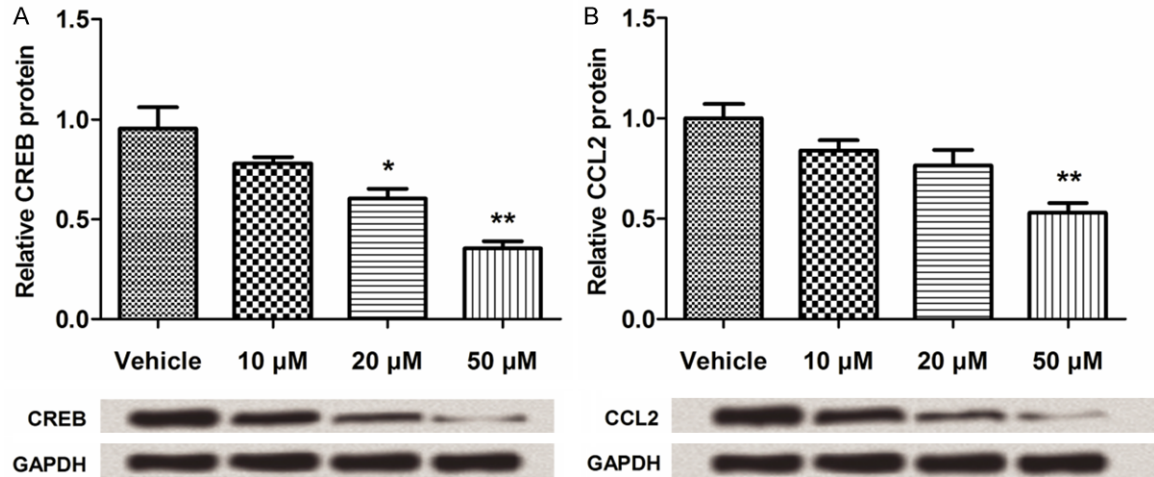


Figure 3. CREB and CCL2 levels were inhibited with increasing concentrations of KG-501 in AOM-induced hepatic encephalopathy model. Protein immunoblots and quantification of (A) CREB and (B) CCL2 immunofluorescence in AOM-treated and AOM + KG-501-treated mouse cortex ($n = 3$). GAPDH acted as an internal control. CREB, cyclic adenosine monophosphate response element binding protein; AOM, azoxymethane; KG-501, CREB inhibitor; * $P < 0.05$ and ** $P < 0.01$ when compared to vehicle-treated mice.

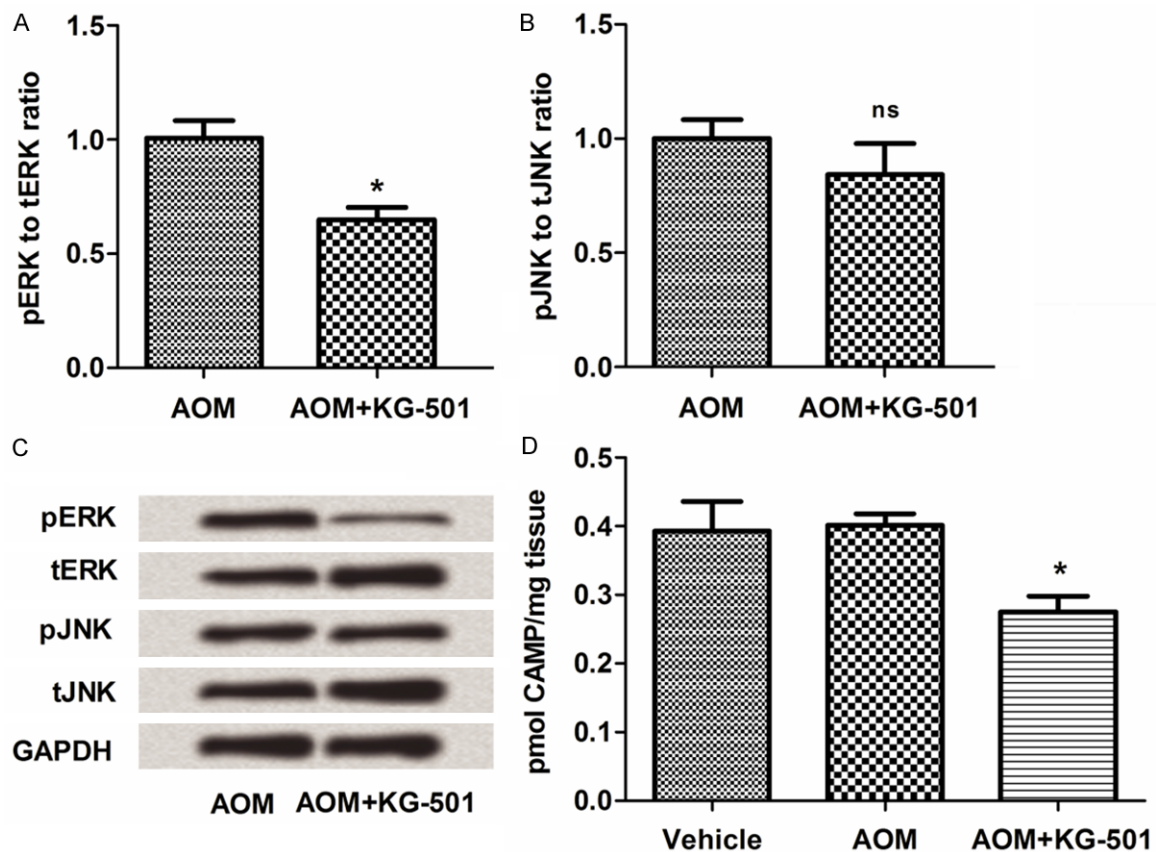


Figure 4. CREB was negative regulation of cAMP/ERK adjustment pathway in AOM-treated mice. Quantification of (A) pERK to tERK, and (B) pJNK to tJNK immunofluorescence in AOM-treated and AOM + KG-501-treated mice cortex ($n = 3$). (C) Protein immunoblots of p/t-ERP and p/t-JNK in AOM and AOM + KG-501 treated mice cortex. GAPDH acted as an internal control. (D) cAMP concentrations in cortex lysates from mice administered vehicle, AOM and AOM + KG-501 ($n = 3$). CREB, cyclic adenosine monophosphate (cAMP) response element binding protein; AOM, azoxymethane; KG-501, CREB inhibitor; pERK, phosphorylated extracellular signal-regulated kinase; tERK, total ERK; pJNK, phosphorylated c-Jun N-terminal kinase JNK; tJNK, total JNK; * $P < 0.05$ when compared to AOM or vehicle-treated mice; ns, no significant difference.

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ERK and JNK were analyzed, the results shown in **Figure 4A-C** suggested that KG-501-mediated signaling lead to the phosphorylation inhibition of ERK, which can be used as a downstream of CREB signaling. But there was no significant difference of JNK in AOM-treated mice with KG-501 injection. Based on these results, we inferred that CREB signaling was required for ERK activation and subsequent produced the proinflammatory chemokine CCL2 following AOM-induced hepatic encephalopathy.

As a measurement of CREB downstream activity, the cAMP in AOM-treated mice cortex with KG-501 injection or not was assessed. We observed decreased level of cAMP in AOM-treated mice infused with KG-501 compared to vehicle and AOM group ($P < 0.05$) as shown in **Figure 4D**. Taken together, the data presented here suggested that activation of the cAMP/CREB pathway may also underlie some of the clinical actions of hepatic encephalopathy.

Discussion

The diagnosis and management of hepatic encephalopathy remain difficult challenges for the medical professionals. It is currently thought that in hepatic encephalopathy the inflammatory stimulus is derived from the systemic circulation; originating from the liver, gut or overt infective source. Cytokines are significant components of the systemic inflammatory response and have recently been associated with progression of hepatic encephalopathy [21, 22]. Pathogenic mechanisms that may be responsible for hepatic encephalopathy have been extensively investigated using animal or central nervous system cell models of hepatic encephalopathy [14]. The consequence of CREB during hepatic encephalopathy had not been investigated previously. This study identified that CREB was present in the cortex of C57BL/6 mice and was upregulated in the brain following AOM-induced acute liver failure. This up-regulation appeared to be protective, as treatment of AOM mice with the CREB inhibitor KG-501 enhanced neurological decline, shorten the time that taken to reach coma, and increased neuroinflammation. In this study, the changes of CREB in hepatic encephalopathy mice cortex and its effects on the neuroinflammation could provide further support for the involvement of multiple factors in the patho-

genesis of hepatic encephalopathy in acute liver failure.

There were substantial evidence suggested the role of proinflammatory mechanisms in the progression of hepatic encephalopathy, neuroinflammation can be regulated by chemokines [23, 24]. Previous research has demonstrated that CCL2, as a small cytokine, could be produced by a number of cell types in the brain, and activate microglia as well as increase the infiltration of circulating macrophages [25, 26]. It has been verified that neuronal CCL2 was upregulated during hepatic encephalopathy and contributed to microglia activation and neurological decline. However, the specific effect of CCL2 on the brain following hepatic encephalopathy due to acute liver failure was not known [16]. In this study, we found that the expression levels of CREB and CCL2 in the cortex of AOM-treated mice with increasing concentrations of KG-501 were decreased. It could be derived that inhibition of CREB reduced the expression of CCL2, and suggested CREB might be involved in neuroinflammation.

In order to reveal the underlying molecular mechanisms by which CREB affected hepatic encephalopathy and involved in neuroinflammation, the phosphorylation levels of ERK and JNK, and the cAMP content in tissue were assessed. As known, mitogen-activated protein kinases (MAPK) are members of a superfamily of serine/threonine protein kinases which were extensively distributed throughout the central nervous system. MAPKs play crucial role in transducing signals to the nucleus, and thereby regulate the genes involved in wide variety of cellular processes [27]. In the three-level cascades of MAPK, ERK1 and ERK2 were the most known extracellular signal-regulated kinases that have been well studied. Recently, a growing body of evidence indicated that the ERK pathway may participate in the neuronal modulation of depression [15, 28]. And also it was clearly known that activation of ERK results in phosphorylation of its downstream target CREB [29]. Our research results showed that the inhibition of CREB caused phosphorylation level of ERK drop, but no significant effect on JNK. Combining with above findings, it could be inferred that CREB affected AOM-induced mice cortex impacting ERK activation, and might therefore affect the expression of CCL2.

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As an important transcription factor, CREB could integrate calcium ions, growth factors and cAMP-induced signal, and CREB activation increased intracellular cAMP level [30]. Recent experiments also confirmed that it could promote CREB binding to DNA by promoting the cAMP aggregation in the cytoplasm of macrophages and upregulation of CREB. However, this effect could be blocked by KG-501, a kind of CREB inhibitors [31]. The experiments have confirmed that cross-talking of ERK and phosphodiesterase-4 regulated cAMP levels by forming cAMP/ERK adjustment pathway, and ERK phosphorylation activated CREB, which played a significant role in neuroprotection and during learning and memory [32, 33]. As our results shown, the KG-501 inhibited CREB, CCL2 expression and ERK phosphorylation, also significantly lowered the cAMP level. It indicated that CREB might contribute to the neuroprotection opposed neuroinflammation through cAMP/ERK adjustment pathway.

In conclusion, we found that CREB was elevated in hepatic encephalopathy mice cortex induced by AOM. CREB played a relief role in hepatic encephalitis, and this adjustment might be made through cAMP/ERK adjustment pathway, and affected CCL2 expression. Further explorations are still needed to focus on the precise role of CREB in the treatment of hepatic encephalopathy.

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Disclosure of conflict of interest

None.

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